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**TITLE: Intracellular Nanoparticle Aggregation as a Mechanism for Inducing Apoptosis in Breast Cancer Cells**

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# **Intracellular Nanoparticle Aggregation as a Mechanism for Inducing Apoptosis in Breast Cancer Cells**

Szu-Wen Wang, University of California, Irvine  
Annual report (September 1, 2009 – August 30, 2010)

## **INTRODUCTION**

The current paradigm of drug delivery using nanotechnology has been focused on coupling chemotherapeutic molecules to nanoparticulate delivery systems. In this work, we propose that pH-triggered aggregation *alone* of nanoparticles (that are non-toxic when not aggregated) can induce death in breast cancer cells. *We hypothesize that non-toxic, protein nanoparticles, when internalized by endocytosis and triggered to aggregate inside breast cancer cells, will be cytotoxic to them.* We will examine (a) the extent to which pH-induced intracellular aggregation is cytotoxic, and (b) whether coupling a chemotherapeutic drug to a pH-responsive protein nanoparticle yields synergistic effects on the toxicity. Although this was a Concept Award which only funded one year of proposed research, a one-year, no-cost extension has been granted to complete the remaining tasks. This current report reflects the one-year update report.

## **BODY**

We are investigating the effects of pH-induced intracellular protein aggregation to breast cancer cells. In this first year of the grant, we have performed tasks and subtasks in all three of the proposed aims that were listed in the Statement of Work. In particular, we have performed the experiments needed to fabricate the control nanoparticles and establish protocols and relevant experimental ranges for proposed assays.

We use a model protein nanoparticle, the E2 subunit of pyruvate dehydrogenase. This protein complex self-assembles from 60 subunits into a 25-nm caged, dodecahedron nanoparticle [3]. E2 and several variants have been reported to be stable over pH 5 - 7.4 and temperatures up to 80°C [3]. In this investigation, we will use a pH-responsive mutant that is stably assembled at pH 7.4 but disassembles and forms aggregates ( $> 1\mu\text{m}$ ) at pH [4]. The 25-nm size of this protein complex is ideal for targeted drug delivery, since receptor-mediated internalization was shown to be most efficient for nanoparticles in the 25-50 nm size range [5]. We expect that when the pH-sensitive E2 variant enters a cell via endocytosis, the decrease in pH will trigger E2 protein aggregation ( $> 1\mu\text{m}$ ) and possibly disrupt the lysosome. Both protein aggregation alone and lysosome disruption is expected to be toxic to the cell.

To test our hypothesis, “control” nanoparticles were first fabricated and characterized to establish baseline effects, for comparison to the pH-dependent aggregating particles. Although we plan to fabricate protein nanoparticles with a ligand previously shown to target several breast cancer lines (peptide LTVSPWY) [1, 2], the first steps were to fabricate the non-targeting nanoparticles. These include: (a) E2-WT (the baseline 25-nm protein nanoparticle scaffold, with no additional changes), (b) D381C (nanoparticle scaffold with an internal mutation for conjugation of doxorubicin, a chemotherapeutic molecule, but no pH-dependent aggregation behavior) and (c) nanoparticles containing the LTVSPWY targeting peptide (with no pH-dependent aggregation behavior). Even though these protein nanoparticles were considered “controls” with respect to the Statement of Work, these investigations have never yet been reported and are of interest for general nanotherapeutic delivery; therefore, the results of these investigations have been prepared for a journal manuscript submission and submitted as conference abstracts. The investigations performed this past year are particularly important to establish baseline effects and also to develop the techniques which are ultimately necessary for our investigations.

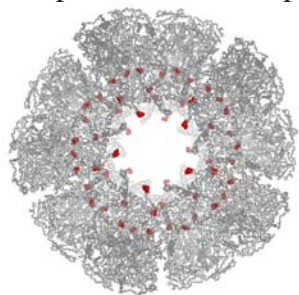
### **Aim 1: Fabricate pH-responsive particles – controls**

***Task 1.1: Protein nanoparticle formation (without targeting ligand)***

***Result: Control D381C protein nanoparticles were fabricated***

Introduction of thiols into the interior cavity of the E2 nanoparticle scaffold enables loading of foreign small molecules dye molecules.[3] In this investigation, we needed to establish that the scaffold can also encapsulate a chemotherapeutic drug, doxorubicin, which is used for treatment of breast cancer. The initial E2 scaffold with native wild-type sequence (E2-WT) has only one native cysteine in each subunit (60 subunits per scaffold), but this cysteine is surface-inaccessible due to its buried location. To encapsulate foreign guest molecules within the scaffold cavity, we mutated the aspartic acid at position 381 of E2 scaffold to cysteine, resulting in 60 thiols within the interior cavity. This mutant scaffold is designated “D381C”.

The gene for the D381C nanoparticle was created using site-directed mutagenesis, and protein expression and purification were performed as previously described.[3]



**Figure 1.** Quaternary protein structure of the D381C nanoparticle, comprising 60 subunits, viewed at the five-fold axis of symmetry. Doxorubicin conjugation sites (amino acid 381) are highlighted in red.

**Task 1.2:** *Protein nanoparticle characterization & confirmation*

**Result:** *Control D381C protein nanoparticles were characterized and confirmed to be structurally intact and thermostable.*

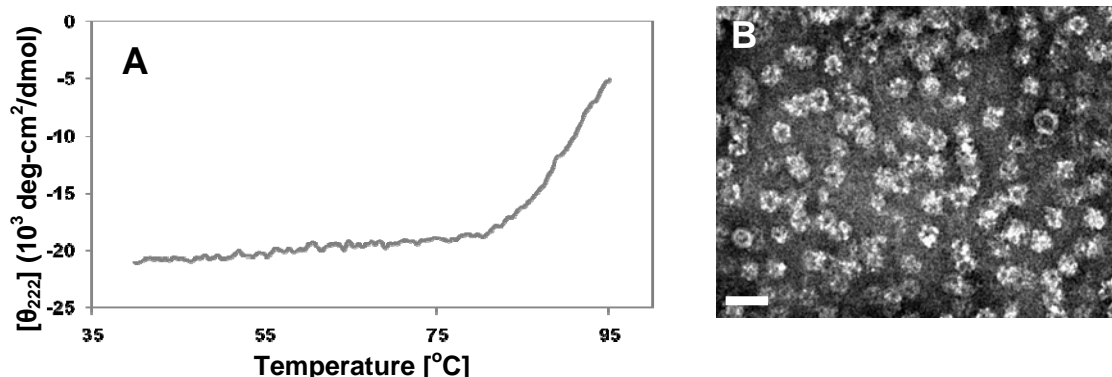
In summary, we confirmed the structure and stability of the D381C protein nanoparticle. Mass spectrometry yielded an average molecular weight of 28,105 Daltons, corresponding to the theoretical value (28,105 Da). CD analysis demonstrated that D381C at pH 7.4 and pH 5.0 are folded with comparable secondary structure and high thermal stability as the baseline wild-type scaffold.[3] Correct 60-mer assembly was also confirmed, with an average hydrodynamic diameter of  $25.4 \pm 0.4$  nm and TEM images showing distinct nanoparticles.

**Task 2:** *Coupling of chemotherapeutic molecules to protein nanoparticle*

**Results:** (a) *Doxorubicin can be coupled to the internal cavity of the protein nanoparticle scaffold.* (b) *D381C protein nanoparticles encapsulating doxorubicin remain intact and stable.*

The 25-nm D381C nanoparticle scaffold has 12 openings which are 5 nm in diameter, allowing for small molecules (e.g., dye, drug) to diffuse into the internal cavity. The maleimide group of DOXO-EMCH is expected to form a thioether linkage through a Michael addition with the surface-accessible thiols at site cysteine-381, which are located in this cavity. We determined the subunit:DOXO-EMCH ratio to range from 1:1.4 to 1:1.9, corresponding to 84 to 114 doxorubicin molecules per scaffold. The covalent coupling of doxorubicin drug molecules to the D381C protein scaffold (D381C-DOX) is confirmed by electrospray mass spectrometry, with a molecular weight 28,856 Da. (Theoretical value is 28,856 Da.)

Our results reveal that conjugation of doxorubicin to D381C (D381C-DOX) yields intact and stable protein nanoparticles. For D381C-DOX, the average hydrodynamic diameter was  $33.8 \pm 4.1$  nm, with the maximum distribution peak at 28.2 nm. The CD analysis of the conjugated scaffold yielded the characteristic profile for a correctly-folded D381C that is still rich in  $\alpha$ -helical content, with the midpoint of unfolding temperature ( $T_m$ ) at approximately 90.5°C (Figure 2A), comparable to the  $T_m$  for unconjugated D381C.[3] TEM confirmed the intact dodecahedral structure of D381C-DOX (Figure 2B). These results show that conjugation of doxorubicin to the protein scaffold does not alter the protein assembly's structure or stability.



**Figure 2.** Thermostability and structural characterization of protein scaffolds encapsulating doxorubicin (D381C-DOX). A. Far-UV circular dichroism thermostability scan at 222 nm revealed  $T_m$  to be 90.5°C. B. Transmission electron micrograph of D381C-DOX. Samples were stained with 2% uranyl acetate. Scale bar is 50 nm.

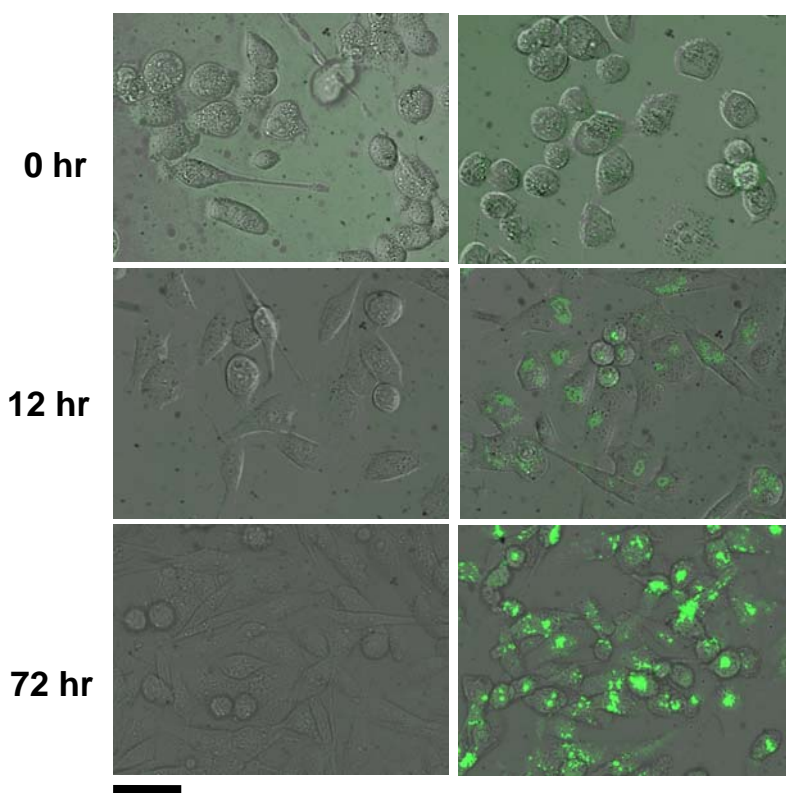
## **Aim 2: Examine nanoparticle endocytosis, compartmental trafficking, and intracellular aggregation of protein nanoparticles**

### ***Task 2.1: Uptake investigations with non-targeted protein nanoparticles***

**Results:** (a) D381C and doxorubicin-coupled protein nanoparticles (D381C-DOX) are internalized by human breast cancer cells. (b) Doxorubicin-coupled protein nanoparticles appear to be taken up by endocytotic pathway.

To investigate cellular uptake of the protein nanoparticles, MDA-MB-231 human breast cancer cells were incubated with D381C labeled with the dye AlexaFluor 532 maleimide (AF532M). Uptake of D381C-AF532M by cells was observed at the first time point investigated (12 hrs), and fluorescence intensity continued to increase over time (Figure 3). However, no observed intracellular fluorescence was apparent for the cells treated with free dye over all incubation times examined.

The images indicate cellular uptake and an accumulation of D381C-AF532M within intracellular compartments, but minimal uptake of free AF532M. This difference between free and conjugated AF532M suggests divergent physical properties and uptake pathways. AF532M is a negatively-charged and hydrophilic fluorescent dye,[6] suggesting low permeability through cell membranes. In contrast, nanoparticles within our size range will be actively endocytosed.[5, 7] Our results show that small molecules which do not naturally cross the cell membrane barrier can, in fact, be delivered into a cell by attachment to the E2 protein nanoparticle.



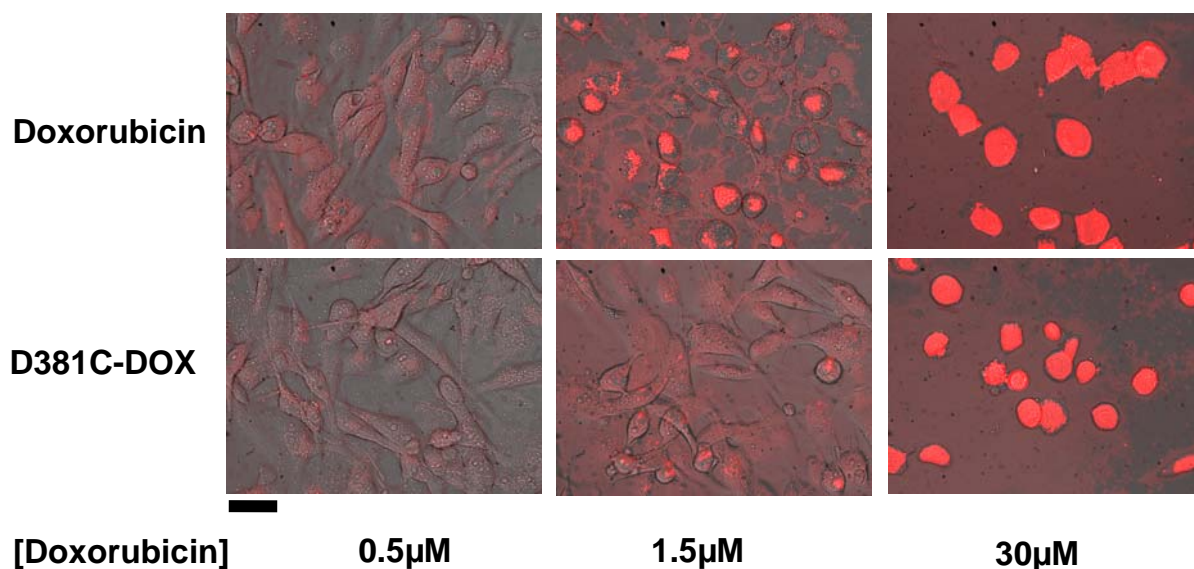
**Figure 3.** MDA-MB-231 cells incubated with (left) free AF532M and (right) protein nanoparticle-conjugated AF532M (D381C-AF532M) at different times. Figures are overlays of phase contrast and fluorescence images. Scale bar is 50  $\mu$ m.

We visualized the uptake of doxorubicin and doxorubicin-coupled protein nanoparticles by fluorescence imaging as well. Figure 4 shows the cellular uptake of free doxorubicin and conjugated doxorubicin (D381C-DOX). As doxorubicin concentration increased, greater intracellular accumulation of the drug was observed. Furthermore, at the low drug concentration, fluorescence appeared to exist more diffusely for cells incubated with free doxorubicin (relative to D381C-DOX) and suggest its presence in the cytoplasm and nucleus.

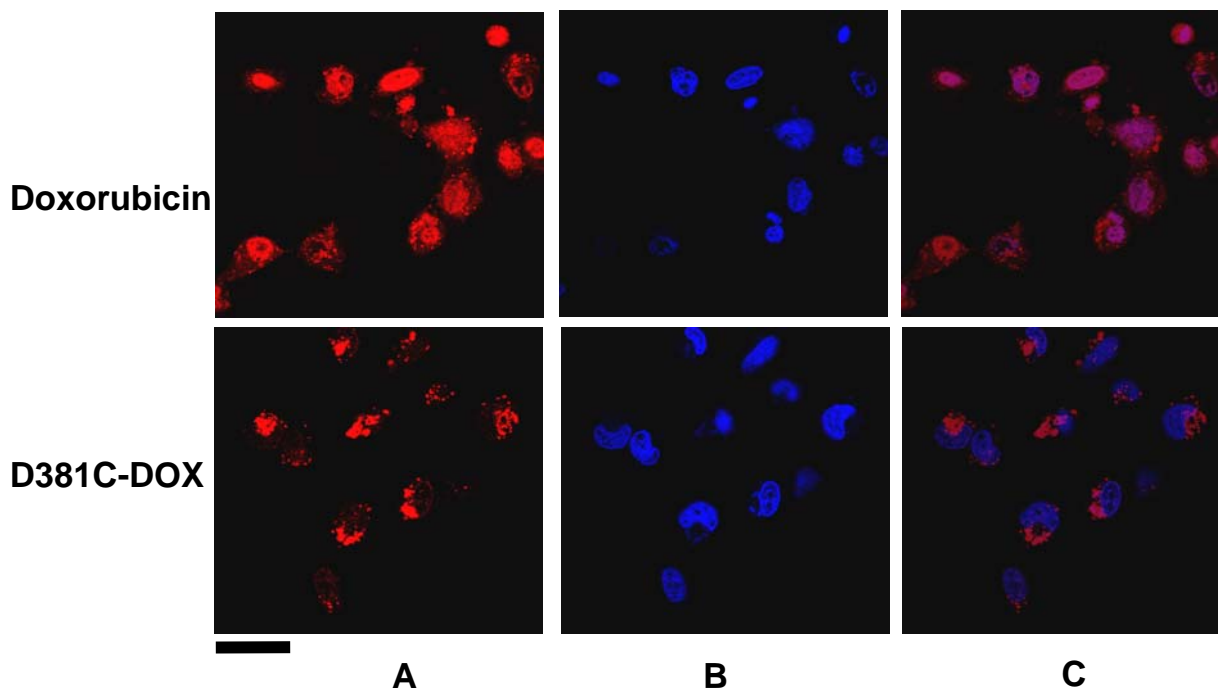
To investigate the intracellular distribution of doxorubicin and D381C-DOX in the MDA-MB-231 tumor cells, we performed confocal laser scanning microscopy (Figure 5). After incubation for two days with free doxorubicin, the drug existed in both the nucleus and in the cytoplasm, with small regions of punctuated accumulation in cellular compartments. In contrast, doxorubicin conjugated to D381C nanoparticles were primarily localized in subcellular vesicles or organelles within the cytoplasm, with only small amounts in the nucleus, which supports endosomal or lysosomal accumulation. These results indicate that free and conjugated doxorubicin use different uptake mechanisms.

Our data is consistent with prior microscopy studies of tumor cells. Other investigators have also reported free doxorubicin to be mainly concentrated in the nucleus while doxorubicin-conjugated serum proteins, polymers, or nanoparticles localize in the cytoplasm or subcellular organelles, such as lysosomes, mitochondria, and the Golgi apparatus.[8-14]





**Figure 4.** MDA-MB-231 cells incubated with (top) free doxorubicin and (bottom) protein nanoparticle-conjugated doxorubicin (D381C-DOX) at different doxorubicin concentrations for 72 hrs. Figures are overlays of phase contrast and fluorescence images. Scale bar is 50 μm.



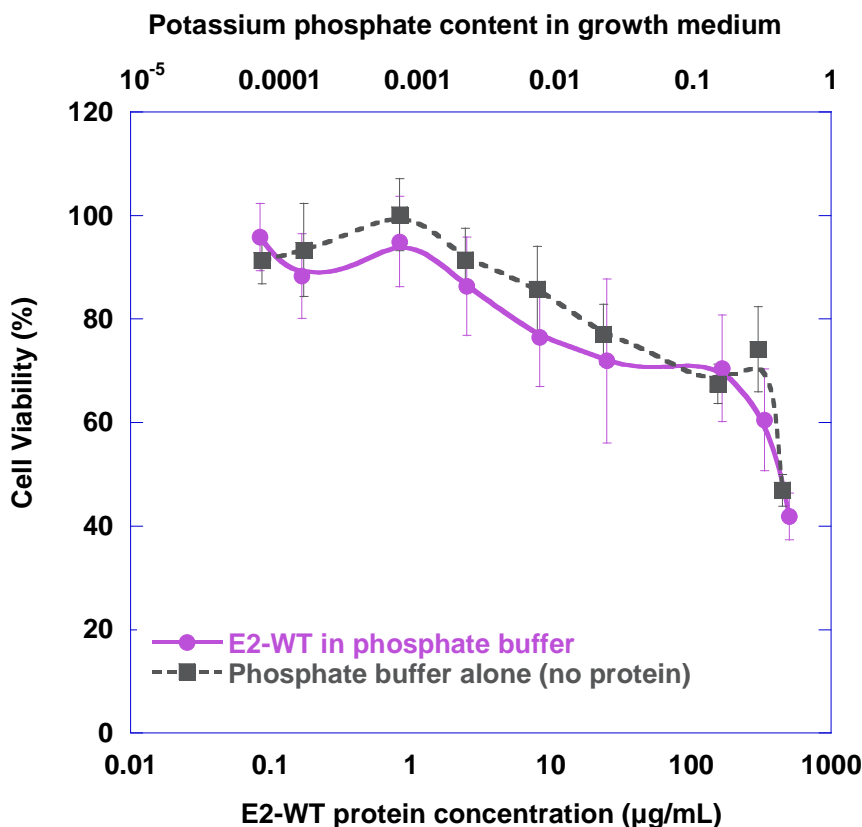
**Figure 5.** Confocal laser scanning microscopy images of MDA-MB-231 cells treated with doxorubicin (top) and D381C-DOX (bottom) at 3 μM doxorubicin-equivalent concentration. (A) Doxorubicin is red, (B) cell nuclei are blue, and (C) overlay of the two images are presented. Scale bar is 50 μm.

### **Aim 3: Determine cytotoxicity of protein aggregates**

**Task 3.1:** Cytotoxicity of non-targeted protein nanoparticles (with and without chemotherapeutic molecules)

**Results:** (a) Non-aggregating protein nanoparticles are not toxic to cells. (b)  $IC_{50}$  of doxorubicin-coupled nanoparticles and of free doxorubicin-alone are  $1.3 \pm 0.3 \mu M$  and  $0.6 \pm 0.01 \mu M$ , respectively.

To investigate the cytotoxicity of the protein nanoparticle scaffold alone, different concentrations of E2-WT ( $0.084 - 502 \mu g/ml$  protein, equivalent to the protein concentrations used in the D381C-DOX studies) were incubated with cells, and the equivalent buffer-alone volume (without protein) was used as a control. The resulting dose response curves for both samples are nearly coincident, with a gradual decrease in cell viability as volume of the sample added was increased (Figure 6). This shows that the protein nanoparticles are not toxic to the cells, but the observed cell death at increasing amounts of protein can be attributed to dilution with buffer. Since increasing the degree of phosphate buffer dilution decreases cell viability at the high dilutions, we attribute this cell death to the exhaustion of nutrients in the medium after such a long incubation time. The lack of toxicity of the E2 protein scaffold itself and the dye-encapsulating scaffold suggest that the E2 nanoparticle can be further pursued as a potential drug

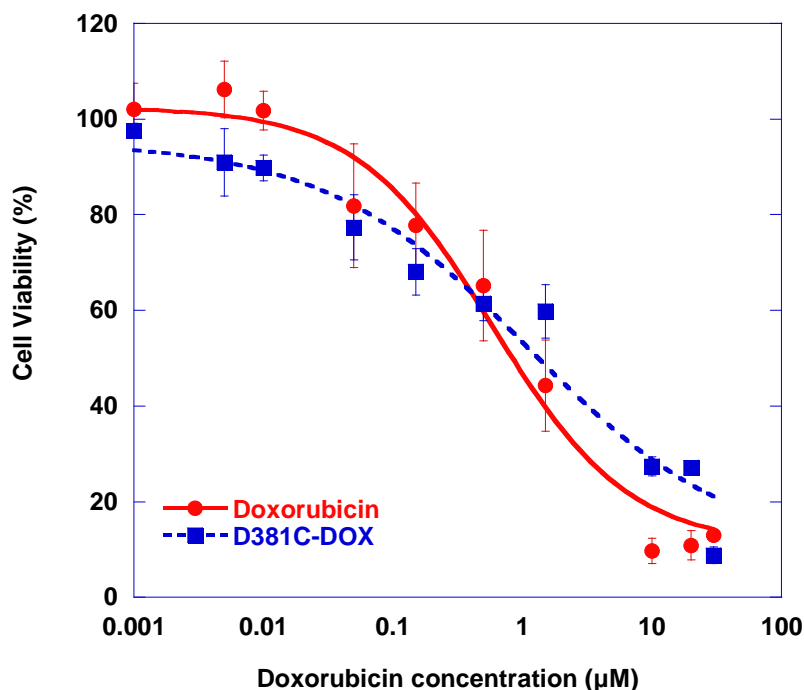


**Figure 6.** Cell viability after incubation with protein scaffold (E2-WT in phosphate buffer) and with equivalent volume of phosphate solution (but no protein) shows no apparent cytotoxicity due to the protein nanoparticle scaffold. The protein concentration of E2-WT is given in the bottom axis, and the dilution degree of phosphate buffer in growth medium is given in the top axis.

delivery and imaging platform, much like that reported for fluorescently-labeled cowpea mosaic virus.[15]

*Cytotoxicity of doxorubicin and D381C-DOX.* The qualitative results observed through fluorescence imaging are consistent with the cell viability data observed after treatment of MDA-MB-231 cells with free and nanoparticle-bound doxorubicin. Using dose-response curves for cells incubated with doxorubicin and D381C-DOX for 72 hours, we determined the  $IC_{50}$  to be  $0.6 \pm 0.01 \mu M$  and  $1.3 \pm 0.3 \mu M$ , respectively (Figure 7). Free doxorubicin is expected to directly diffuse through the lipid bilayers into the cell and nucleus.[16, 17] In contrast, our slightly higher level of toxicity for free doxorubicin relative to conjugated doxorubicin can be explained by the additional steps required for D381C-DOX to be taken up by the cells through the endocytic pathway, with gradual pH change and corresponding drug release from the nanoparticle, and escape from the endosome/lysosome compartments into the cytosol and nucleus. Hence, it is likely that 72 hrs may be an insufficient period for the complete release of doxorubicin from the D381C scaffold.

These  $IC_{50}$  values are within the range of typical values obtained in other types of nanoparticle-based delivery systems. Prior literature has reported  $IC_{50}$  of doxorubicin in MDA-MB-231 to range between  $0.1 \mu M$  and  $2 \mu M$  for cells incubated 48 to 72 hr.[18-20] Investigations for doxorubicin conjugated to various types of nanoparticles have reported both higher[10, 11, 20-22] and lower[12, 23, 24]  $IC_{50}$  values relative to free drug.



**Figure 7.** Dose response curves of MDA-MB-231 cells incubated with free doxorubicin and protein nanoparticle-conjugated doxorubicin (D381C-DOX) for 72 hrs.  $IC_{50}$  values are  $0.6 \pm 0.01 \mu M$  for doxorubicin and  $1.3 \pm 0.3 \mu M$  for D381C-DOX.

## **KEY RESEARCH ACCOMPLISHMENTS**

- The potential of intracellular molecular delivery with anti-cancer therapeutic was examined.
- E2 protein nanoparticles with drug conjugation abilities were fabricated.
- E2 protein nanoparticles conjugated with drug doxorubicin were characterized and confirmed to be structurally intact and thermostable.
- Non-aggregating protein nanoparticles were demonstrated to be non-toxic to cells, which shows the potential of the nanoparticle scaffold itself to be used in imaging and therapeutic delivery.
- IC<sub>50</sub> of doxorubicin-coupled nanoparticles and of free doxorubicin-alone are  $1.3 \pm 0.3 \mu\text{M}$  and  $0.6 \pm 0.01 \mu\text{M}$ , respectively.
- Investigations support the uptake of the protein nanoparticles via the endocytic pathway, which shows the potential of using the pH-responsive behavior of the  $\Delta\text{N}$  form of E2 (aggregates at pH 5) for inducing cell death via intracellular protein aggregation.

## **REPORTABLE OUTCOMES**

### Manuscripts:

“Protein Nanocapsules Containing Doxorubicin as a pH-Responsive Delivery System,” Dongmei Ren, Felix Kratz, and Szu-Wen Wang. In preparation, as of 8/30/10. (Submitted to journal *Small*, as of 12/15/10)

### Results presented at conferences and presentations:

- “Protein Nanoparticles Encapsulating Doxorubicin as a pH-Dependent Drug Delivery System,” Dongmei Ren, Szu-Wen Wang, Society for Biological Engineering conference, San Francisco, January 2011, abstract submitted. (Abstract accepted as of 12/15/10)
- “Drug-Encapsulating Protein Nanoscaffold as a Controlled Release Delivery System,” Dongmei Ren, Merce Dalmau, Szu-Wen Wang, American Chemical Society National Meeting, Anaheim, March 2011, abstract submitted. (abstract accepted as of 12/15/10)
- Brookhaven National Laboratory, Upton, NY. 2010 Joint NSLS and CFN Users’ Meeting, Workshop on Self-Assembly of Soft and Biomolecular Materials: Structure, Dynamics, and Interactions. May 2010. (Invited workshop presentation)
- University of California, Irvine, Department of Chemical Engineering and Materials Science. May 2010. (Invited Seminar)
- University of Delaware, Center for Molecular and Engineering Thermodynamics & Delaware Biotechnology Institute (co-sponsored). April 2010. (Invited seminar)

### Educational degrees and student training supported by this award

- Dongmei Ren, Ph.D. student, Chemical and Biochemical Engineering Program, UC Irvine
- Nicholas Molino, Ph.D. student, Chemical and Biochemical Engineering Program, UC Irvine

## CONCLUSIONS

This investigation has probed, for the first time, the effects of E2 protein nanoparticles on breast cancer cells and their potential in intracellular molecular delivery. We showed that the E2 scaffold containing accessible thiol groups is able to bind the 6-maleimidecaproyl hydrazone derivative of doxorubicin within its hollow cavity. Fluorescence imaging showed AF532M and doxorubicin can be internalized by breast cancer cells when coupled to D381C. Free doxorubicin distributed mainly into the nucleus and cytoplasm of cells, while D381C-DOX was localized in subcellular organelles, suggesting the conjugated doxorubicin was taken up by endocytosis. Our results further reveal that doxorubicin bound through an acid-sensitive linker within the scaffold will indeed induce cell death. Collectively, these properties demonstrate that virus-like protein nanoparticles represent a promising new opportunity for nanomaterials in therapeutic delivery and imaging applications. Future work includes coupling targeting ligands and the pH-responsive aggregation behavior of these protein nanoparticles to these current nanoparticles, to assess the feasibility of using the intracellular protein aggregation as a means for inducing additional cell death.

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## APPENDICES

None